Production and characterization of a broad-specificity polyclonal antibody for *O,O*-diethyl organophosphorus pesticides and a quantitative structure–activity relationship study of antibody recognition

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ABSTRACT

Polyclonal antibody (PAb) with broad-specificity for *O,O*-diethyl organophosphorus pesticides (OPs) against a generic hapten, 4-(diethoxyphosphorothioyloxy)benzoic acid, was produced. The obtained PAb showed high sensitivity to seven commonly used *O,O*-diethyl OPs in a competitive indirect enzyme-linked immunosorbent assay (ciELISA) using a heterologous coating antigen, 4-(3-(diethoxyphosphorothioyloxy)phenylamino)-4-oxobutanoic acid. The 50% inhibition value (IC$_{50}$) was 348 ng mL^{-1} for parathion, 13 ng mL^{-1} for coumaphos, 22 ng mL^{-1} for quinalphos, 35 ng mL^{-1} for triazophos, 751 ng mL^{-1} for phorate, 850 ng mL^{-1} for dichlofenthion, and 1301 ng mL^{-1} for phoxim. The limit of detection (LOD) met the ideal detection criteria of all the seven OP residues. A quantitative structure–activity relationship (QSAR) model was constructed to study the mechanism of antibody recognition using multiple linear regression analysis. The results indicated that the frontier-orbital energies (energy of the highest occupied molecular orbital, E_{HOMO}, and energy of the lowest unoccupied molecular orbital, E_{LUMO}) and hydrophobicity (log of the octanol/water partition coefficient, $\log P$) were mainly responsible for the antibody recognition. The linear equation was $\log(\mathrm{IC}_{50}) = -63.274E_{HOMO} + 15.985E_{LUMO} + 0.556\log P - 25.015$, with a determination coefficient (r^2) of 0.908.

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1. Introduction

Organophosphorus pesticides (OPs) have been widely used in agricultural and domestic applications for more than six decades [1]. However, reports of OP intoxication in humans and animals have increased public concern [1]. As an alternative to the laborious and expensive instrumental methods used to quantify OPs, immunochemical analysis such as enzyme-linked immunosorbent assays (ELISAs) have been successfully developed as a semiquantitative or quantitative screening tool for detecting OPs [2–4]. Immunoassays are rapid, cost-effective, have high-throughput, and in some cases are capable of working on-site or remotely on-line.

Immunoassays are often developed to recognize a single target with high specificity. However, sometimes immunoassays are developed that can detect various related compounds in one simple test, and these immunoassays are called broad-specificity, generic, group- or class-specific, or multi-analyte assays [5–7].

A broad-specificity immunoassay can be developed by raising numerous antibodies that recognize individual targets and then incorporate all the antibodies into a single test. But the most commonly used method to produce a broad-specificity immunoassay is to produce an antibody having broad-specificity by using a "generic hapten", which should exhibit common features of all target analytes [6–9]. The first step of immunoassay development is the design of a hapten. However, the antibody specificity resulting from the newly designed hapten is often unpredictable, and this knowledge comes only after time-consuming and laborious animal experiments. Sometimes, an apparent rationally designed "generic hapten" is unable to generate antibodies with the desirable specificity and sensitivity [10]. Sudi et al. [11] and Banks et al. [12] attempted to develop broad-specificity assays for OPs, but the produced broad-specificity polyclonal antibodies had unsatisfac-

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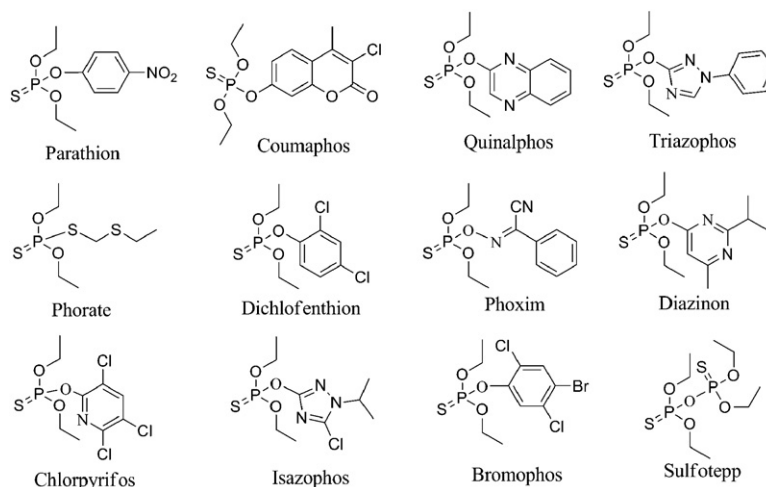*E-mail addresses:* sydscau@yahoo.cn (Y.-D. Shen), ymsun@scau.edu.cn (Y.-M. Sun).

Fig. 1. Molecular structures of 12 O,O-diethyl OP compounds.

tory sensitivity. Later attempts made by other immunochemists had great improvements in sensitivity, but the broad-specificity characteristics or uniform responses to all the analytes of interest were still not desirable [13–16].

The difficulty in developing broad-specificity immunoassays lies with the approach used for the basis of assay design. It is hoped that the hapten required when developing an immunoassay is designed using a rational basis. But the lack in understanding of the specific interactions between antibodies and haptens or target analytes has been the most restricting in overall assay development. In recent years, computational techniques, like quantitative structure–activity relationship (QSAR), have been applied to help to design a broad-specificity immunoassay [17]. From the results of cross-reactivity (CR) and molecular modeling studies, one can understand better what structural and electronic features are important for antibody binding [18,19], and that information can be used in an interactive process to improve hapten design or improve antibody recognition [17]. In the work described here, a novel generic hapten containing a thiophosphate moiety and a benzene ring was synthesized and used for generating broad-specificity polyclonal antibodies for O,O-diethyl OPs (Fig. 1). The influence of hapten heterology on antibody sensitivity was also studied. Furthermore, a QSAR model was used to study the mechanism of antibody recognition, and hopefully the QSAR results may be useful in future studies for improving the ELISA properties.

2. Experimental

2.1. Reagents and materials

Neat pesticide standards were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). 3-Aminophenol, O,O-diethyl phosphorochloridothioate, and 4-hydroxybenzoic acid were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shenyang, China). Bovine serum albumin (BSA), dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), ovalbumin (OVA), 3,3′,5,5′-tetramethylbenzidine (TMB), and complete and incomplete Freund's adjuvants were purchased from Sigma (St. Louis, MO, USA). N,N-Dimethylformamide (DMF), methanol, succinic anhydride, and Tween-20 were obtained from Damao Chemical Reagent Co., Ltd. (Tianjin, China). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was obtained from Boster Biotech Co., Ltd. (Wuhan, China). Thin-layer chromatography (TLC) was performed on 200 mesh, 2.5 mm precoated silica gel (F254) on glass sheets and

was obtained from Qingdao Haiyang Chemical Co., Ltd. (Qingdao, China). Polystyrene ELISA plates were obtained from Jiete Biotech Co., Ltd. (Guangzhou, China). All other chemicals and organic solvents were of analytical grade.

Buffers used in this study were prepared as follows: 50 mmol L^{-1} carbonate buffer (pH 9.6) for coating plates, 10 mmol L^{-1} PBST solution (10 mmol L^{-1} phosphate buffer saline (PBS, pH 7.4) containing 0.1% Tween-20, pH 7.4) was used for washing plates, 0.1 mol L^{-1} citrate and sodium phosphate buffers (pH 5.4) was used for the substrate buffer, and 2 mol L^{-1} H_2SO_4 was used as the stopping reagent. TMB was initially dissolved in DMF (15 mg mL^{-1}), and 150 μL of this DMF solution and 2.5 μL of 6% (w/v) H_2O_2 were added to 10 mL of substrate buffer to obtain the TMB solution.

2.2. Instruments

Ultraviolet–visible (UV–vis) spectra were recorded on a UV-160A Shimadzu spectrophotometer (Kyoto, Japan). High-resolution mass spectrometry (HRMS) analyses were performed using a MAT95XP high-resolution mass spectrometer (Thermo, USA). Nuclear magnetic resonance (NMR) spectra were obtained with both the DRX-400 and DRX-600 NMR spectrometers (Bruker, Germany-Switzerland). ELISA plates were washed using a Multiskan MK2 microplate washer (Thermo Labsystems, USA). Absorbance was measured at a wavelength of 450 nm using a Multiskan MK3 microplate reader (Thermo Labsystems, USA).

2.3. Hapten synthesis and characterization

The synthetic routes used to produce the immunizing hapten (hapten 1) and the coating hapten (hapten 2) are shown in Figs. 2 and 3, respectively. UV–vis, HRMS, and NMR spectra were obtained to characterize the synthesized haptens.
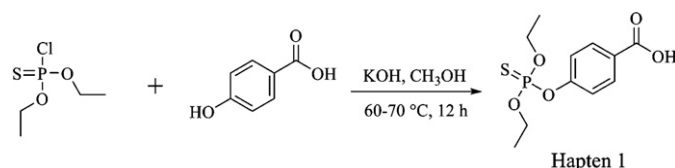

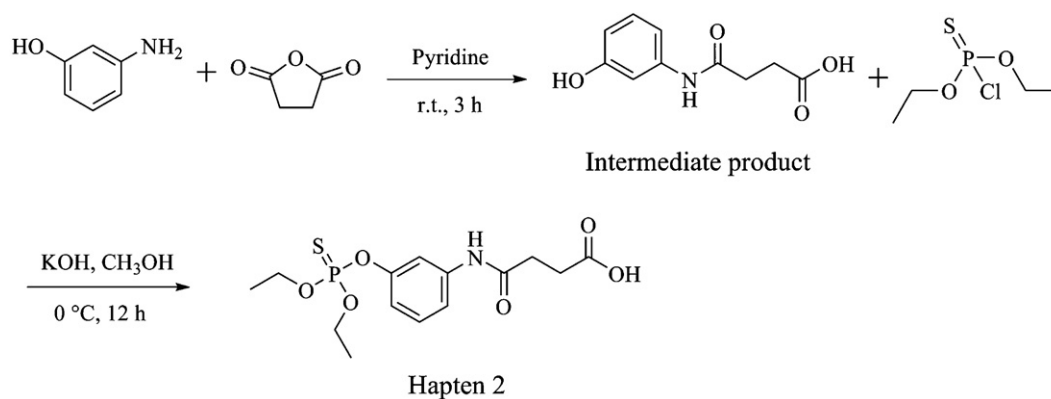Fig. 2. Immunizing hapten (hapten 1) synthetic scheme.

Fig. 3. Plate coating hapten (hapten 2) synthetic scheme.

2.3.1. Synthesis of hapten 1

O,O-Diethyl phosphorochloridothioate (1.81 g, 9.6 mmol) was added dropwise to a stirred mixture of 4-hydroxybenzoic acid (0.884 g, 6.4 mmol), KOH (0.898 g, 16 mmol) and methanol (50 mL). After stirring for 12 h at 60–70 °C, the mixture was filtered, and the solvent was removed under reduced pressure. The residue was diluted with ethyl acetate and extracted into a 2 mol L^{-1} NaOH solution. The solution was acidified with 6 mol L^{-1} HCl. 1.05 g of 4-(diethoxyphosphorothioyloxy)benzoic acid (hapten 1) was obtained as a white solid in 56.4% yield. HRMS (EI) calculated for $C_{11}H_{15}O_5P_1S_1$ [M] 290.0372, found 290.0374. ^{1}H NMR (400 MHz, CDCl$_3$ and TMS) δ: 1.36 (t, J = 7.1 Hz, 6H, CH$_3$); 4.24 (qd, J = 10.0, 7.1 Hz, 4H, CH$_2$); 7.26 (d, J = 8.1 Hz, 2H, ArH); 8.09 (d, J = 8.5 Hz, 2H, ArH). ^{13}C NMR (150 MHz, CDCl$_3$ and TMS) δ: 15.86 (d), 65.28 (d), 120.88 (d), 126.67, 132.01, 154.89 (d), 171.45. ^{31}P NMR (242 MHz, CDCl$_3$ and TMS) δ: 62.18.

2.3.2. Synthesis of hapten 2

3-Aminophenol (5.46 g, 0.05 mol) was dissolved in pyridine (10 mL) and added dropwise to a stirred solution of succinic anhydride (5 g, 0.05 mol) in 10 mL pyridine. After stirring for 3 h at room temperature, 20 mL saturated K$_2$CO$_3$ was added to the mixture and then extracted with ethyl acetate. The water phase was acidified by adding 6 mol L^{-1} HCl to give 3.5 g of 4-(3-hydroxyphenylamino)-4-oxobutanoic acid (intermediate product) as a white solid in 33.5% yield. HRMS (EI) calculated for $C_{10}H_{11}O_4N_1$ [M] 209.0683, found 209.0683. ^{1}H NMR (400 MHz, d$_6$–acetone and TMS) δ: 2.65 (s, 4H, CH$_2$); 6.52 (ddd, J = 7.8, 2.2, 0.9 Hz, 1H, ArH); 7.01 (d, J = 8.1 Hz, 1H, ArH); 7.07 (t, J = 8.0 Hz, 1H, ArH); 7.34 (s, 1H, ArH); 9.1 (s, br, 1H, NH). ^{13}C NMR (150 MHz, d$_6$–DMSO and TMS) δ: 28.83, 31.08, 106.15, 109.73, 110.08, 129.31, 140.33, 157.58, 169.96, 173.88.

O,O-Diethyl phosphorochloridothioate (1.13 g, 6 mmol) was added dropwise to a stirred mixture of the intermediate product (1.31 g, 6.27 mmol), KOH (1.01 g, 18 mmol), and methanol (60 mL). After stirring for 12 h at 0 °C, the mixture was filtered, and the solvent was removed under reduced pressure. The residue was subjected to column chromatography (silica gel, CHCl$_3$/MeOH (6:1)) to give 0.6 g of 4-(3-(diethoxyphosphorothioyloxy)phenylamino)-4-oxobutanoic acid (hapten 2) as a yellow oil in 27.6% yield. HRMS (EI) calculated for $C_{14}H_{20}O_6N_1P_1S_1$ [M] 361.0743, found 361.0737. ^{1}H NMR (400 MHz, CDCl$_3$ and TMS) δ: 1.32 (t, J = 7.3 Hz, 6H, CH$_3$); 2.60 (t, J = 6.3 Hz, 2H, CH$_2$); 2.69 (t, J = 6.2 Hz, 2H, CH$_2$); 4.19 (qd, J = 9.6, 7.1 Hz, 4H, CH$_2$); 6.87 (d, J = 7.5 Hz, 1H, ArH); 7.16–7.23 (m, 2H, ArH); 7.48 (s, 1H, ArH); 8.16 (s, 1H, NH). ^{13}C NMR (150 MHz, CDCl$_3$ and TMS) δ: 15.82 (d), 29.34, 31.64, 65.16 (d), 112.65 (d), 116.54, 129.65, 138.95, 150.85 (d), 170.89, 177.16. ^{31}P NMR (242 MHz, CDCl$_3$ and TMS) δ: 62.55.

2.4. Preparation of hapten–protein conjugates

Hapten 1 was coupled to BSA to be used as an immunogen, and both hapten 1 and hapten 2 were coupled with OVA by the active ester method to be used as plate coating antigens. Hapten (12 μmol), NHS (14.4 μmol) and DCC (14.4 μmol) were dissolved in 1000 μL of DMF. The mixture was stirred gently at 4 °C overnight, and then centrifuged at 10,956 × g for 5 min. The supernatant (900 μL) was added dropwise to BSA (136 mg) or OVA (90 mg) in 9 mL PBS (pH 7.4). The conjugation mixture was stirred at 4 °C for 12 h and then purified by gel filtration on Sephadex G-25. The eluted conjugates were dialyzed against water and then freeze-dried before storage at 4 °C. UV–vis spectral data were used to confirm the structures of the final conjugates and the ratios of haptens to carrier proteins were determined by using the 2,4,6-trinitrobenzene sulfonic acid (TNBS) method [20].

2.5. Preparation of polyclonal antibodies

Two New Zealand male rabbits (designated OPs-1258 and OPs-1259, supplied by the Guangdong Medical Laboratory Animal Center) weighing 1.5–2.0 kg were immunized subcutaneously at multiple sites (12–15 sites) on the back with a 1:1 mixture (v/v, 1000 μL) of hapten 1–BSA conjugate (1 mg) in 0.01 mol L^{-1} PBS (500 μL, pH 7.4) and Freund's complete adjuvant. Three weeks after the initial injection, booster immunizations were given intraperitoneally with the same amount of immunogen emulsified with incomplete Freund's adjuvant. The booster immunizations were given every 3 weeks. Rabbits were bled using the ear vein 1 week after each injection. Blood samples were left to coagulate for 1 h at about 25 °C and then overnight at 4 °C, and centrifuged at 10,956 × g for 10 min to obtain the antiserum. The antiserum was purified using ammonium sulfate precipitation [21] to produce the PAb, which was divided into aliquots and stored at −20 °C until used.

2.6. Optimized ELISA procedure

Using a chequerboard titration, all incubations were performed at 37 °C except for the coating antigen. Flat-bottom polystyrene ELISA plates were coated with hapten 1–OVA (1 μg mL^{-1}, 100 μL well^{-1}) in carbonate buffer (pH 9.6) overnight at 4 °C. The wells were washed 5 times with PBST solution, and then blocked with 5% skim milk in PBS buffer (200 μL well^{-1}) for 1 h. After washing 5 times with PBST solution, the wells were incubated with 100 μL of diluted antibody in PBST for 1 h and washed 5 times with PBST solution. HRP-conjugated goat anti-rabbit IgG diluted 1:9000 in PBST was added (100 μL well^{-1}). After incubation for 1 h and washing 5 times with PBST solution, TMB solution was added

Table 1
Symbols for the QSAR descriptors and their definition.

Symbol	Definition
E_{HOMO}	Energy of the highest occupied molecular orbital
E_{LUMO}	Energy of the lowest unoccupied molecular orbital
$\log P$	Log of the octanol/water partition coefficient
VM	Molecular volume
μ	Dipole moment
α	Molecular polarizability
R	Refractivity
q_P	Charge on the P atom
E_{hyd}	Hydration energy
A_a	Surface area (approx.)
A_g	Surface area (grid)
M_w	Molecular weight

to the wells (100 µL well^{-1}) and incubated for 15 min. The reaction was stopped by addition of 50 µL well^{-1} of 2 mol L^{-1} H$_2$SO$_4$, and the absorbance was recorded at 450 nm.

In a competitive indirect ELISA (ciELISA), hapten 1–OVA and hapten 2–OVA were both used as coating antigens to investigate the effect of hapten heterology on sensitivity of the ELISA. Flat-bottom polystyrene ELISA plates were coated with hapten 2–OVA (1 µg mL^{-1}, 100 µL well^{-1}) in carbonate buffer (pH 9.6) overnight at 4 °C. The wells were washed 5 times with PBST solution, and blocked with skim milk as described above. OP standards dissolved in PBS containing 10% methanol (50 µL well^{-1}) and PAb diluted with PBST (50 µL well^{-1}) were added, incubated for 1 h, and washed 5 times with the PBST solution. HRP-conjugated goat anti-rabbit IgG diluted 1:9000 in PBST was added (100 µL well^{-1}). After incubation for 1 h and washing 5 times with PBST solution, TMB solution was added to the wells (100 µL well^{-1}) and incubated for 15 min. The reaction was stopped by addition of 50 µL well^{-1} of 2 mol L^{-1} H$_2$SO$_4$, and the absorbance was recorded at 450 nm. Competitive curves were obtained by plotting the normalized signal (B/B$_0$) against the logarithm of analyte concentration. The 50% inhibition value (IC$_{50}$) and limit of detection (LOD) were obtained from a four-parameter logistic equation of the Sigmoidal curves using Originpro 7.5 software. The percentage of CR was calculated according to the following equation: (IC$_{50}$ (parathion, nmol mL^{-1})/IC$_{50}$ (cross-reactant, nmol mL^{-1})) × 100.

2.7. Theoretical modeling

The structures of all compounds were initially drawn in Hyper-Chem 7.0 software (Hypercube, Inc., USA). Molecular structures were then fully optimized using Hartree-Fock (HF) with a 6-31G* basis set in the Gaussian 03 software. The hydrophobic, electronic, quantum-chemical, as well as geometrical parameters (Table 1) were extracted from the Gaussian files or obtained by HyperChem 7.0 based on the optimized structures. Subsequently, all the descriptors were then taken as possible predictor variables in the multiple linear regressions for the outcome measure log(IC$_{50}$) as calculated by SPSS 13.0 software (SPSS, Chicago, USA).

3. Results and discussion

3.1. Hapten design and synthesis

Hapten design and synthesis plays a key role in developing immunoassays [22–24]. For a broad-specificity immunoassay, selection of a hapten having features common to all structures within the group to be targeted is required. Most of the O,O-diethyl OPs have both an aromatic ring and a thiophosphate ester moiety. Previous work by Jang et al. [15] showed that a hapten containing both of these two moieties could produce antibodies with desirable properties for use in class-specific determinations.

Based on careful observation of the OP molecular structures and the considerations mentioned above, a hapten (hapten 1) was designed containing both an O,O-diethyl thiophosphate and a phenyl ring, which also contained a carboxyl linker at the para position of the phenol (Fig. 2). The synthesis was straight forward using O,O-diethyl phosphorochloridothioate and 4-hydroxybenzoic acid as starting materials (Fig. 2). The plate coating antigen also plays a key role in ELISA sensitivity. Heterology in the coating conjugate often results in weaker antibody affinity providing higher sensitivity to the analytes [25], and heterologous coating antigens have been used to improve the sensitivity of ELISA methods [15,16,26–28]. Therefore, a coating conjugate (hapten 2) containing both O,O-diethyl thiophosphate and a phenyl ring, but a different longer-linker (4-amino-4-oxobutanoic acid) at the ortho position was synthesized by a two step reaction (Fig. 3). The synthesized haptens were characterized by UV–vis spectrometry, HRMS, and NMR.

Two different strategies have been used to conjugate OP haptens to carrier proteins: first, attachment to the phosphate ester backbone [29] and second, direct attachment to the carrier protein through the phosphorus atom [30]. The second method could potentially expose most of the phosphate ester and hence, the second method was used in this work. The synthesized conjugates demonstrated qualitative differences between the carrier protein and conjugate in the UV–vis spectra, suggesting successful hapten conjugation to the carrier protein. The hapten coupling ratios with the carrier proteins were 30.6, 9.3, and 8.9 for hapten 1–BSA, hapten 1–OVA, and hapten 2–OVA, respectively.

The large difference in the coupling ratio between hapten 1–BSA and hapten 1–OVA might be caused by the different number of reaction sites on BSA and OVA. It was suggested that BSA possesses a total of 59 lysine ε-amine groups (with only 3–35 of these typically available for derivatization), while OVA contains only 20 lysine ε-amine groups capable of conjugation [31]. Theoretically, there are fewer sites to interact with the antibody when OVA is used as the coating antigen, thereby producing better sensitivity than using a coating antigen with a higher load of hapten.

3.2. Antiserum titer

Antisera from two rabbits injected with hapten 1–BSA were collected 1 week after the 3rd booster injection and tested for the presence of antibody recognizing the immunizing hapten by a chequerboard titration (Fig. 4). Antiserum OPs-1259 showed a higher titration than antiserum OPs-1258 did. The titer values after the 3rd injection were about 1.0 at 1/20,000 dilution and 1/180,000
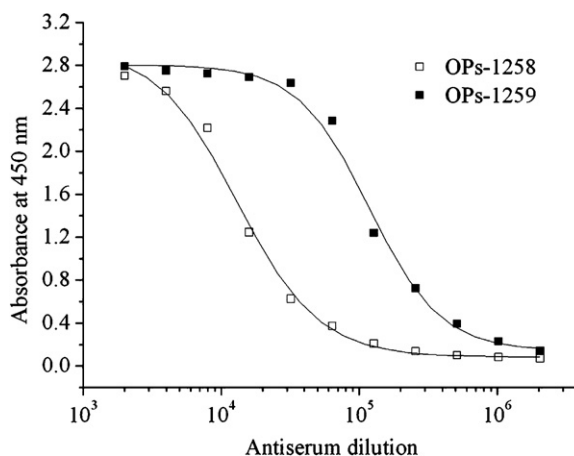
Fig. 4. Chequerboard titration of two polyclonal antisera. Microtitration plates were coated with 1 µg mL^{-1} of hapten 1–OVA.

Table 2
ELISA[a] cross-reactivity for 12 O,O-diethyl OPs against PAb-1259.

Compounds	Coating antigen					
	Hapten 1–OVA			Hapten 2–OVA		
	IC_{50} (ng mL^{-1})	LOD (ng mL^{-1})	CR (%)	IC_{50} (ng mL^{-1})	LOD (ng mL^{-1})	CR (%)
Parathion	12,402	1,598	100.0	348	39	100.0
Coumaphos	1,017	107	1520.7	13	0.2	2975.0
Quinalphos	8,395	1,331	151.3	22	3	1700.0
Triazophos	6,442	672	207.1	35	2	1081.8
Phorate	19,644	7,755	56.4	751	28	41.3
Dichlofenthion	5,349	1,734	250.9	850	4	44.1
Phoxim	589	209	2161.4	1,301	37	27.3
Diazinon	20,729	4,078	62.5	7,939	983	4.6
Chlorpyrifos	82,812	21,505	18.0	13,484	6640	3.1
Isazophos	18,072	2,753	73.9	14,257	1197	2.6
Bromophos	28,230	5,684	59.4	24,873	5630	1.9
Sulfotepp	98,282	28,579	14.0	39,893	5428	1.0

LOD = limit of detection; CR = cross-reactivity. Percentage of CR was calculated according to the following equation: (IC_{50} (parathion, nmol mL^{-1})/IC_{50} (cross-reactant, nmol mL^{-1})) × 100.

[a] All reactions were performed in triplicate on microtitration plates coated with coating antigen at 1 μg mL^{-1}.

dilution for antiserum OPs-1258 and OPs-1259, respectively. Thus, antiserum OPs-1259 was purified producing PAb-1259, and PAb-1259 was used in subsequent studies.

3.3. Characterization of polyclonal antibodies

The specificity of the obtained PAb was evaluated by performing competitive assays using 12 commonly used O,O-diethyl OPs (Fig. 1), and the obtained IC_{50} values (nmol L^{-1}) were used to calculate the CR. The results were displayed in Table 2. The PAb showed broad-specificity towards all the OPs. The CR results were in agreement with what might have been predicted by using an immunogen that exhibited both a thiophosphate ester and an aromatic ring, which were present in most of the tested OPs. The CR values were high for parathion, coumaphos, quinalphos triazophos, dichlofenthion, and phoxim, but they were relatively low for phorate, diazinon, chlorpyrifos, isazophos, bromophos, and sulfotepp. Based on careful analysis of the OP molecular structures, phorate, and sulfotepp consist primarily of only the thiophosphate ester moiety, while diazinon, chlorpyrifos, isazophos and bromophos have bulky aromatic substituents (such as three chlorine or bromine atoms or alkyl substituents). The relatively low CR of the antibody to these OPs might be due to their unique molecular structures.

Although the antibody showed high CR towards all OPs in the homologous ELISA, the sensitivity was undesirable. A heterologous ELISA is commonly used to eliminate the problems of unwanted CR cause by strong affinity of the antibodies to the spacer arm that leads to no or poor inhibition by the target compound [32]. Therefore, the conjugate, hapten 2–OVA, was chosen for the plate coating antigen. The results corroborate the use of heterologous assays for improved sensitivity (Table 2). The IC_{50} values for parathion, coumaphos, quinalphos, phorate, triazophos, dichlofenthion and phoxim were significantly improved in the heterologous ELISA, and the LOD values satisfied the current criteria for monitoring these OP residues.

In contrast, Jang et al. [15] used O,O-diethyl O-(5-carboxy-2-fluorophenyl) phosphorothioate as the immunogen, and demonstrated that the obtained monoclonal antibodies showed high specificities for bromophos-ethyl, chlorphyrifos-ethyl, dichlofenthion and parathion-ethyl. These antibodies also had uniform specificities for the O,O-dimethyl OP pesticides, bromophos-methyl, chlorpyrifos-methyl, and parathion-methyl, but had much lower specificities for diazinon and azinphos-methyl [15]. It might be suggested that introducing a fluorine atom on the phenyl ring of the immunogen contributed greatly to the recognition of bromophos-ethyl and chlorophyrifos-ethyl. However, no data was presented in their paper showing relative specificities of their antibody for coumaphos, quinalphos, triazophos, and phorate, which also may have been influenced by the introduction of the fluorine substituent.

Several antibodies have been obtained from generic haptens that exhibit broad-specificity for OPs, but the overall sensitivity or broad-specificity of these antibodies were not desirable [6–16]. In this study, the obtained antibody exhibited broad-specificity to seven commonly used OPs and had good sensitivity while demonstrating a uniform response. This antibody will be evaluated to be used as a qualitative or semiquantitative screening tool for OPs to ensure the safety of food.

3.4. Antibody recognition study

Antibody–antigen interactions are fundamental to immunoassay [33]. However, the interactions at the molecular level are in general undetermined. An understanding of these specific interactions can help to improve the affinity and specificity of immunoassays by aiding in re-designing the hapten or optimizing ELISA conditions (coating antigen, tracer, pH, ionic strength, etc.). Quantitative structure–activity relationship (QSAR) studies are used to correlate a biological response with structural parameters. QSAR has been applied to study the epitopes of 14 structurally similar fluoroquinolone (FQ) antibiotics recognized by an anti-FQ monoclonal antibody [34]. In this study, structural parameters (such as charge, molecular weight, and volume) of 12 O,O-diethyl OPs were obtained using theoretical calculations and correlated with OP inhibition values (IC_{50}) to PAb-1259 (in a heterologous ELISA). Table 1 summarizes and defines the predictor variables used in this work.

Table 3
QSAR model[a] for modeling the inhibitory activity on PAb-1259.

Regression equation	r^2	s	F
$\log(IC_{50}) = -63.274 E_{HOMO} + 15.985 E_{LUMO} + 0.556 \log P - 25.015$	0.908	0.433	26.195

[a] The overall quality of the model is indicated by the determination coefficient, r^2, the standard deviation, s, and the Fisher's statistic, F.

Table 4
Values of QSAR descriptors calculated for the organophosphorus pesticides.

Compounds	E_{HOMO} (eV)	E_{LUMO} (eV)	$\log P$	$\log(IC_{50}{}^a)$
Parathion	−0.3544	0.0607	3.760	0.0755
Coumaphos	−0.3233	0.0664	4.030	−1.3979
Quinalphos	−0.3268	0.0686	3.721	−1.1549
Triazophos	−0.3288	0.1073	2.918	−0.9586
Phorate	−0.3305	0.1415	3.533	0.4594
Dichlofenthion	−0.3411	0.1108	5.085	0.4314
Phoxim	−0.3413	0.1166	3.940	0.6395
Diazinon	−0.3480	0.1198	3.583	1.4165
Chlorpyrifos	−0.3476	0.0770	5.157	1.5850
Isazophos	−0.3425	0.2244	2.47	1.6574
Bromophos	−0.3443	0.0962	5.822	1.8002
Sulfotepp	−0.3485	0.1964	3.964	2.0926

^a $\log(IC_{50})$ values were calculated using units of nmol L^{-1} for IC_{50}.

The resulting QSAR model is given in Table 3 together with the regression statistical parameters, and values used for the variables are listed in Table 4. It is generally accepted that a model with a determination coefficient value greater than 80% ($r^2 \geq 0.80$), a large F, and a small s has good predictability [35]. Therefore, by this criterion the obtained QSAR model calculated here had good predictability. The plot of the predicted $\log(IC_{50})$ values based on the derived equation versus experimental data is shown in Fig. 5. Fig. 5 shows that the predicted $\log(IC_{50})$ values are in good agreement with the experimental data. The $\log(IC_{50})$ exhibited a reasonable correlation with the three-parameter (E_{HOMO}, E_{LUMO} and $\log P$) equation in the QSAR model. This three-parameter equation suggests that if the correct changes were made in the antigen, there would be an increase in the E_{HOMO} value or a decrease in the E_{LUMO} or $\log P$ values, resulting in enhanced activity of the antibody.

It is suggested that the antigen–antibody recognition is based on steric criteria and on interactions resulting from the electronic properties of the molecules [24,34,36]. In our work, the results from the QSAR model indicated that the frontier-orbital energies and hydrophobicity are mainly responsible for antibody recognition of these OPs. E_{HOMO} is a rough measure of the electron-donating ability of a compound and, normally, increasing its value can increase the biology activity, while the E_{LUMO} acts in reverse [37]. It was clear from the graphical representation of the HOMO and LUMO for parathion (Fig. 6, analogous graphical representations were also
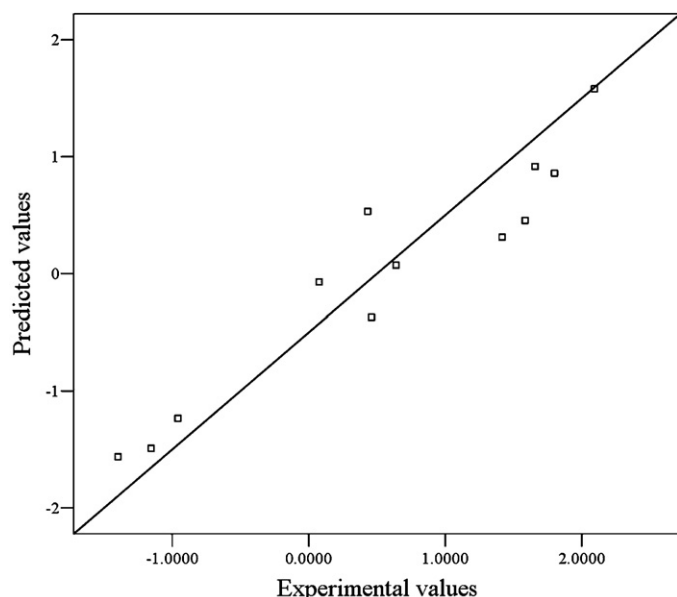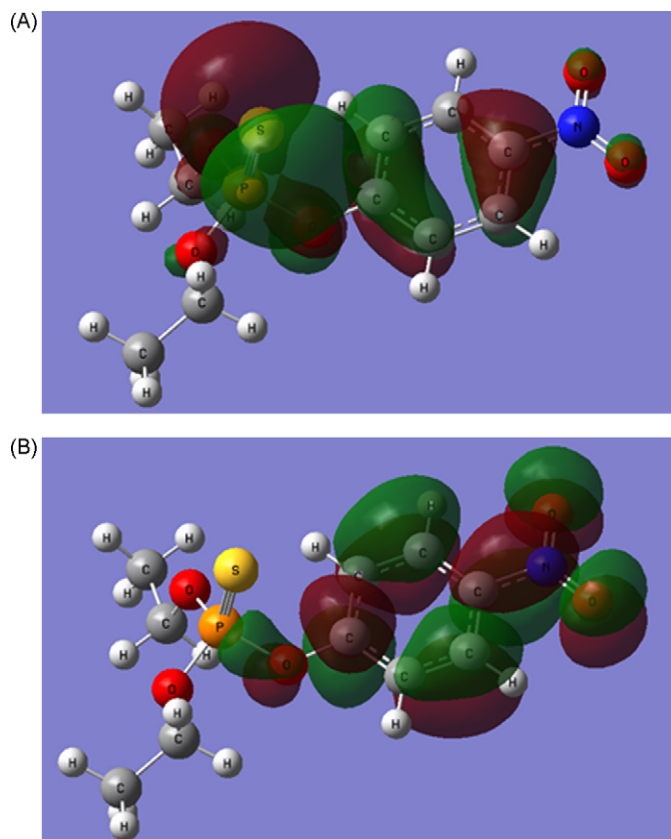
Fig. 6. The graphical representation of the HOMO (A) and LUMO (B) for parathion.

obtained for other analytes, data not shown), that the thiophosphate ester was most likely an important portion of the OPs epitope for antibody recognition. Increasing the electron-donating ability of the thiophosphate ester may enhance antibody recognition, or decreasing the electron-withdrawing ability of the thiophosphate ester may also increase antibody affinity. The frontier-orbital energies and hydrophobicity of a compound can play an important role in biological activity [37], and results obtained from the three-parameter equation indicates that the more hydrophobic a compound is, the less it will bind the antibody. However, none of our data suggested that a steric criteria was responsible for antibody recognition, which may be due to the small difference in volume of the O,O-diethyl OPs.

According to the above discussion, the obtained QSAR model can be used to predict antibody affinity for OP related analogs. Also, the information from the model may be useful for re-designing a heterologous coating hapten. In this work, spacer arm heterology of the coating conjugate and linkage position to the hapten were used to improve sensitivity of the ELISA. Kim et al. [25,38] and Harrison et al. [39] suggested that heterology in the structure of the coating hapten and linkage position produced a more remarkable improvement in the sensitivity of the ELISA than that of heterology in the spacer arm length. Therefore, based on the QSAR model it may be possible to re-design a coating hapten to produce weaker recognition for the antibody. A newly re-designed hapten may then result in better assay sensitivity, and we are currently pursuing this line of research.

4. Conclusions

With the aim of developing a broad-specificity immunoassay for O,O-diethyl organophosphorus pesticides (OPs), we produced a polyclonal antibody against a generic OP hapten and examined

Fig. 5. Plot of predicted $\log(IC_{50})$ values versus experimental $\log(IC_{50})$ values.

the characteristics of the antibody with a ciELISA. The antibody showed broad-specificity and high-sensitivity to seven commonly used O,O-diethyl OPs. Based on the determined IC_{50} values, and the hydrophobic, electronic, quantum-chemical, and geometrical molecular parameters of all analytes obtained by molecular modeling, a QSAR model was constructed using multiple linear regression analysis. The QSAR model involved three descriptors: energy of the highest occupied molecular orbital (E_{HOMO}), energy of the lowest unoccupied molecular orbital (E_{LUMO}) and log of the octanol/water partition coefficient ($\log P$). The results of the QSAR model suggested that increasing the E_{HOMO} or decreasing the E_{LUMO} or $\log P$ might improve antibody recognition. This information may be useful for improving the immunoassay features by re-designing the hapten or by optimizing the ELISA conditions. Studies are on-going to apply the information generated here.

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